

Maize *Adh1* as a Monitor of Environmental Mutagens

by Michael Freeling*

The maize *Adh1* gene is introduced, with citations. The use of this gene as a mutagen monitor is discussed, with emphasis on differentiating quantitative and qualitative parameters. A proper assessment of hazard from environmental pollutants requires data on possible mutagen specificities for different kinds of DNA.

Introduction

The value of a biological monitor of environmental mutagens depends on the mutagenicity parameter to be measured. The environment may be assessed for the presence of mutagenic activity, the quantity of mutagenic activity, the molecular nature of the mutagens, or the molecular nature of the induced lesions and their genetic consequences.

This paper has two objectives. First, I will present relevant results and citations on the maize alcohol dehydrogenase-1 gene (*Adh1* gene; ADH enzyme, EC 1.1.1.1.) and its product. The use of *Adh1* as a qualitative as well as quantitative monitor of mutagenicity will be discussed. Second, what little is known as to the nature of the gene in higher organisms, and my assumption that lesions in regulatory DNA components rather than structural gene components may preferentially cause birth defects and cancer, are woven into an argument for developing qualitative genetic monitors of the environment. Both of these objectives lend themselves to propagandizing. It is extraordinarily difficult to obtain data which prove that particular pollutants do or do not cause birth defects or cancer in people.

The *Adh1* Gene and Its Product

In 1966, Schwartz (1), and Scandalios (2) reported finding ADH allozyme variants. Schwartz (1, 3) used these variants to define the *Adh1* gene, which was then mapped on the long arm of chromosome 1,

within 2 map units of *Knotted* and *lemon-white*. This gene specifies the major ADH activity of the quiescent embryo (largely scutellum, a storage organ) and the pollen grain. ADH is dimeric, with a MW variously estimated at 60,000 or 80,000 (4, 5). The abbreviation used for this enzyme is ADH1·ADH1. Such a descriptive nomenclature is necessary to avoid confusion with other dimeric ADH isozymes present in other tissues, or in response to anaerobic conditions (6).

The ADH1·ADH1 enzyme is stable, relatively easy to purify, and is amenable to virtually all of the techniques of protein chemistry. Being in maize, *Adh1* benefits from a distinguished history and, consequently, established cytogenetic protocol.

ADH in Pollen Grains

A typical maize plant sheds 10^7 monoploid, trinucleate gametophytes. Using pollen shed from plants of the genotype *Adh1*⁺/*Adh1*⁻, where the negative mutant fails to express product, we developed an *in situ*, cytochemical stain for pollen ADH. The result was that half of the pollen grains stained yellow and translucent (ADH⁻) while the other half stained blue and opaque (ADH⁺) (7), as shown in Figure 1.

Owing to the availability of about two dozen ethyl methanesulfonate-induced, biochemically characterized, *Adh1*⁻ mutants (from Drew Schwartz, largely unpublished; method of recovery given in ref. 8), I was able to quantify reversion and intragenic recombination frequencies (7, 9). Several million pollen grains are collected under rigorously controlled conditions from each of several homoal-

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Nature of the Gene in Higher Organisms

For the purposes of this paper, a "gene" is defined as a single, simple complementation group, synonymous with the original meaning of the word "cistron" (12). The gene is composed of a structural gene component, containing the information for the primary structure of the polypeptide and adjacent *cis*-acting regulatory gene component(s). Such a gene is diagrammed in Figure 2. The evidence for the existence of *cis*-acting receptors within the gene is still fragmentary (13-15).

Maize contains about 4 pg of DNA in an unrepliated, monoploid nucleus (16). If maize contains 10,000 structural genes, about twice the number of lethal complementation groups (or chromomeres on polytene chromosomes) in *Drosophila melanogaster*, and if each structural gene, plus that DNA which encodes the message leader sequence, is taken to be 1300 nucleotide pairs, then there would be 1.3×10^7 nucleotide pairs, or 0.0013 pg of structural gene in the monoploid nucleus. The percent structural gene is simply calculated as $0.0013/4 = 0.1\%$ of the DNA in the genome. Even if this calculation were off by an order of magnitude, the conclusion is inevitable: only a small part of the DNA in maize (or any other higher organism) is structural gene component. It is tempting to endow some of

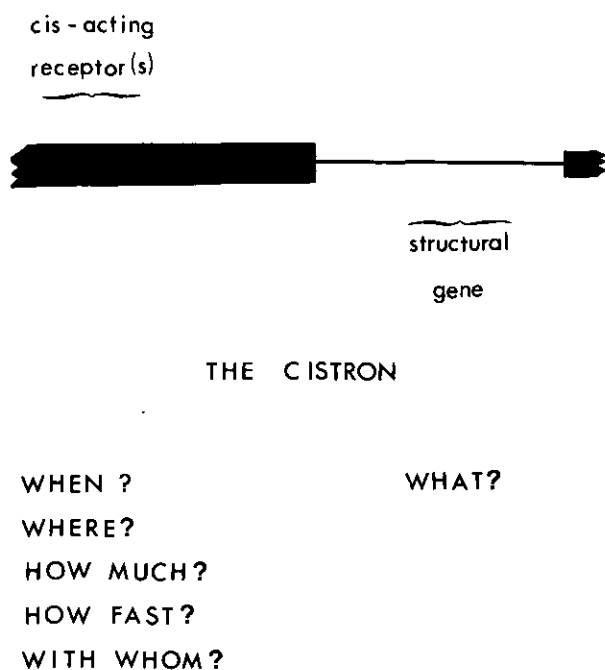


FIGURE 2. The "gene" in higher organisms is at least bipartite. The open-ended black boxes contain DNA, about which we know little. Functions for gene components are followed by a "?".

this mysterious DNA with regulatory functions since structural genes certainly do not control their own behavior; their behavior must include the classical embryological phenomena of determination, competence and somatic heredity.

There is sound but limited evidence that point-mutagens do not induce detectable mutants in *cis*-acting regions of the gene (17, 15), although such regulatory variants certainly occur spontaneously, and are probably induced by certain types of chromosomal breakage events (15, 18, 19). Since all mutagens do not have an equal ability to mutate regulatory gene components, mutagen monitors which detect structural gene components alone could miss other types of mutagens specific to regulatory gene components. For a fanciful example, insertion sequences specific to certain classes of intermediate-repeat DNA would be undetectable in reversion systems.

Forward Mutant Frequencies at *Adh1*

The advantage of monitoring forward mutant frequency is that all lesions capable of eliminating or lowering gene expression are scored. At its present state of development, maize *Adh1* could be used as a mediocre forward mutant monitor. The difficulty is technical; since about 2% of normal pollen is either aborted or ADH^- in its staining reaction, it is necessary to germinate pollen before staining. These methods have been developed; spontaneous, forward mutant frequency ($Adh1^+ \rightarrow ADH^-$ viable pollen grain) for two of the common *Adh1* isoalleles is below 2×10^{-7} (20). All pollen is germinated on newly cut slices from solid medium modified from the recipe of Cook and Walden (21). This method is called "David's Bread Loaf"; our high percent germinations and reduction of density effects on germination depend on this new method. A forward mutant is recognized as a rare ADH^- gametophyte with a pollen tube surrounded by ADH^+ grains. The necessity of germinating pollen before staining reduces the resolution of this system to about 10^{-6} , and requires much more work than reversion assays.

As will be seen in the sections which follow, the major advantage of a forward mutation monitor is not only unbiased detection, but also our ability to recover and analyze extensively *Adh1*-defective mutants.

Chemical Selection of *Adh1*-Defective Mutants via Pollen and Their Recovery

Because higher plants have gametophytic stages where haploid nuclei are expressed and which filter

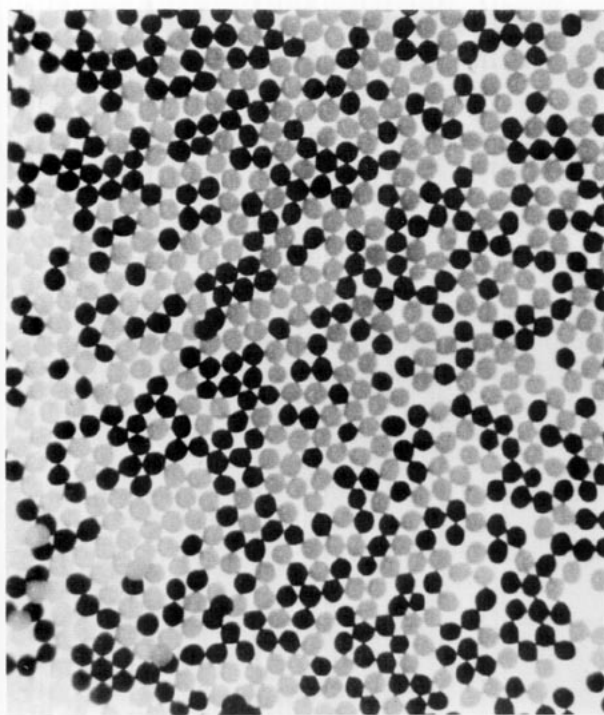


FIGURE 1. Pollen segregating *Adh1*⁺ and *Adh1*⁻ after being stained specifically for ADH activity. The darker grains are ADH⁺; each gametophyte is approximately 90 μ m in diameter.

lelic *Adh1*⁻ mutant plants. The total number of grains is estimated by using techniques of glycerine suspension and counting on Millipore filter grids. The number of blue grains—phenotypic revertants—is counted directly by screening all immobilized grains. Homoallelic ADH⁺ frequencies were well below 10^{-5} . On the other hand, ADH⁺ frequencies from heteroallelic mutant pairs (if they originate from the same progenitor isoallele) ranged from 2×10^{-5} to 10^{-3} .

***Adh1* as a Quantitative Monitor of Environmental Mutagens**

Since ADH may be stained in pollen grains after they are shed, the resolution of pollen analysis is below 10^{-7} . For this reason, reversion of any of the two dozen known, biochemically characterized (9), *Adh1*⁻ mutants could be monitored, as well as the frequency of gene conversion generated by any of the hundreds of possible heteroallelic pairs. In these systems, significant elevation of ADH⁺ frequencies over control values would be taken as evidence for (a particular kind) of mutagenic activity.

Practically speaking, 2-month-old potted plants would be positioned about an environment suspected to contain a volatile environmental mutagen.

Several different point-mutants and several heteroallelic pairs should be used, and each plant should be paired with a sibling which remains at a control site or under control conditions. Pollen from 2.5–2.75 month plants might be collected directly in the field, or immature tassels could be moved under heat lamps for forced shedding. One person may collect from 50 plants in 1 hr; the total freezing-staining process could consume less than 3 man-hours; the stained pollen may be stored indefinitely. Counting is done in a laboratory, with each estimate taking 30–45 min. This system is not confined to testing volatiles; seeds or seedling could be planted directly into suspect soil, but proper controls could pose difficulties.

There are at least two drawbacks to using the maize *Adh1* gene as a quantitative monitor, apart from the general pitfalls of reversion assays. For most purposes, the *His* reversion system in *Salmonella*, developed by Ames and associates (10) or similar microbial systems, seems preferable because the exact molecular nature of the point, intragenic mutants (e.g., GC transitions, deletion frame-shift) has been established. However, since crop plants may convert innocuous chemicals into stable mutagens which at least have a chance to contaminate our food sources (11), there are cases where crop plant systems are required. It is not clear how many mitoses intervene between the zygote and a mature pollen grain because germ-cell determination happens late and erratically during development. In addition, the stages of meiosis are not synchronous within a tassel. Therefore, calculation of true mutation rates (*Adh1*⁻ \rightarrow ADH⁺ \cdot cell-generation⁻¹) is not possible. Since different developmental stages are known to be associated with different propensities for mutation, repair and recombination, if environmental mutagenic activity were not evenly and continuously present, the *Adh1* system could generate much noise. There is no question that maize *Adh1*, along with other plant germ cell systems, is simply more difficult to quantify meaningfully as compared to microbial test systems.

Reversion Analyses Are Biased

Monitors which utilize gene reversions exclusively suffer from a major shortcoming: only lesions capable of reverting the original mutant sites are detected. Since point-mutants are generally recognized and chosen based on altered enzymes or polypeptides, reversion tests are biased toward the structural gene components of the genome.

out gross chromosomal abnormalities, we are able chemically to select rare, transmissible, *Adh1*-defective mutants from among millions of wild-type pollen.

ADH⁻ pollen grains survive concentrations of allyl alcohol ($C=C-OH$) vapor which kill almost all ADH⁺ gametophytes (22-24). This chemical selection system has been used to recover *Adh1*-deficient mutants which arose spontaneously and which were induced with ionizing radiation (15). Our procedure is diagrammed in Figure 3. Central to this protocol is to mark the male (target) and female (tester) parents with different electrophoretically detectable *Adh1* alleles. This allows each presumptive mutant F_1 to be analyzed for ADH1:ADH1 allozymes; slivers of scutellum are extracted without lowering the viability of the F_1 seed. Mutants are confirmed on the basis of aberrant allozyme profiles (Fig. 3).

Genetic and Biochemical Tests to Which *Adh1*-deficient Mutants May be Subjected

After a prospective *Adh1*⁻ mutant pollen grain is recovered in rare F_1 seeds, the seed is analyzed for ADH1 allozyme profile (Fig. 3). A sliver of scutellum is extracted and subjected to electrophoresis in starch gel; this process does not kill the seed. On the basis of the allozyme profile, the *Adh1*⁻ mutant may be placed into one of several classes (15): haploid, *Adh1*-dysfunction, underproducer, overproducer, charge change of product, low stability of homodimer only, and others. After this primitive protein level classification, mutants may be subjected to one or more of the tests which follow.

If the *Adh1*⁻ mutant is dysfunctional in all organs tested (scutellum, root and pollen), then genetical tests are most valuable.

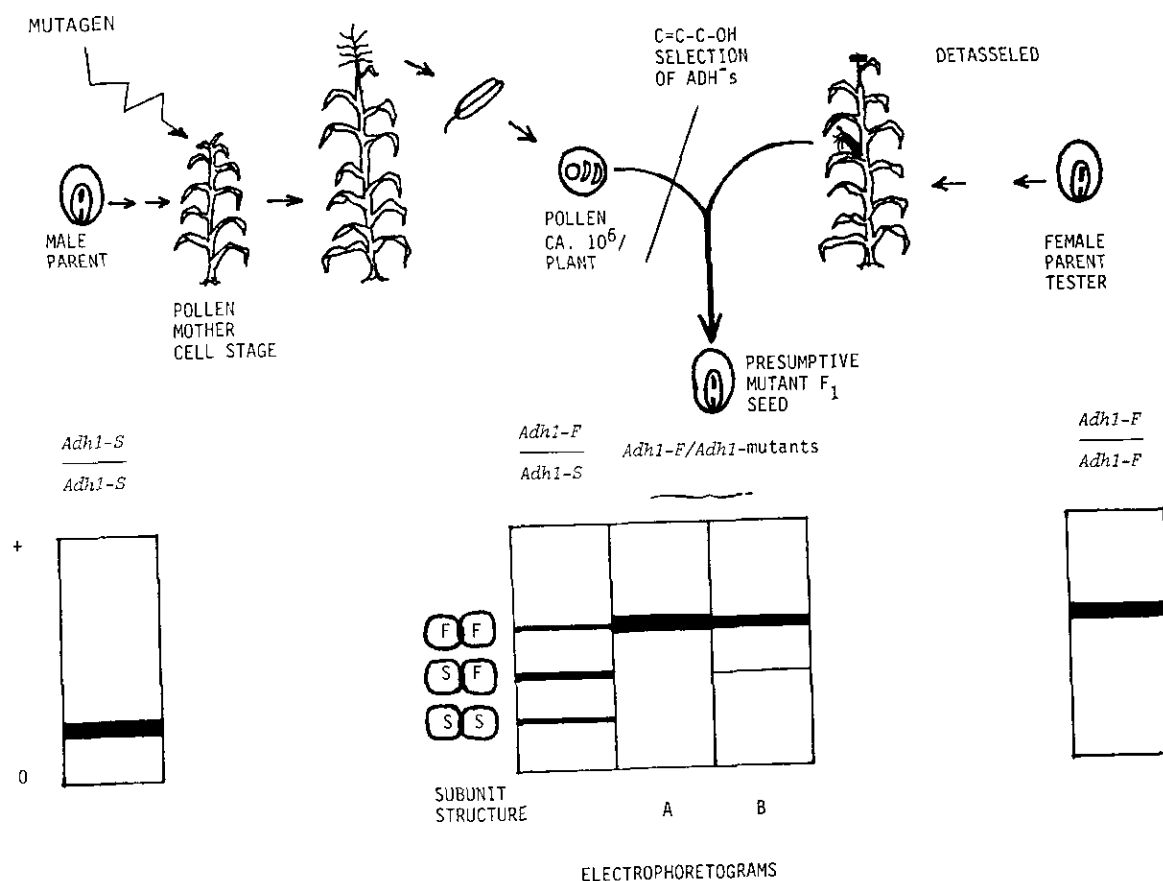


FIGURE 3. Scheme for induction, selection, recovery, and confirmation of *Adh1*-S-defective mutants. The electrophoretograms depict ADH1:ADH1 allozyme profiles obtained from scutellar extracts; (0) origin; (+) anode. The male parent also carries one or more unique genetic markers in order to identify haploids and contaminants.

F₁ Pollen Abortion, % Transmission through Male and Female, and Survival of Mutant Homozygote. Since *Adh1* function is fully dispensable, a lesion confined to the structural gene should transmit fully through either gametophyte. Small chromosomal aberrations—as well as reciprocal translocations, inversions, etc.—are often recognized by lowered transmission frequencies or characteristic amounts of pollen abortion (25, 26).

Chromosomal Analyses of F₁ Plants. Root tip mitotic chromosome counts test aneuploidy involving extra centromeres. Pollen mother cells may be examined at pachytene for chromosome identification and pairing configurations, diakinesis for chromosome counts and detection of transpositions, and at the anaphases for bridges. There are major cytogenetic advantages to working with maize (27). These advantages are particularly relevant since every clear case for an artificially induced regulatory mutant in higher organisms seems to involve chromosome breaks (15).

Frequency of Revertants and Their Analysis. Pollen analysis, as described previously in this paper, is able to resolve reversion frequencies at 10^{-7} . Deletions of *Adh1* should be nonrevertible. Insertional mutants, on the other hand, might be highly revertible.

We have not yet developed a reliable chemical selection scheme permitting the recovery of rare ADH⁺ revertants or recombinants in a background of ADH⁻ gametophytes.

Intragenic Recombinational Tests. The new mutant could be crossed with a series of known EMS-induced point-mutants within *Adh1* and assessed for ability to recombine intragenically using methods described previously. A short deletion might be expected to recombine with some of these point-mutants, but not all; rationale of deletion mapping is from Benzer (12).

If some ADH product is made by the mutant allele, then conventional enzymology and protein chemistry can be used to detect a primary structure difference and, thus, a difference in the structural genes.

Electrophoretic Mobility, Thermolability, K_m, and V_m. If the mutant ADH1 subunits are altered in these functional parameters, a damaged structural gene component would be indicated. Laughner (28) and Felder et al. (4) have worked out several of these methods. (It should be noted that all enzyme kinetics data reported for maize ADH in the acetaldehyde to ethanol direction have used an unacceptable buffer: Tris-HCl.)

Dissociation of Dimers to Monomers and Reassociation Back to Active Dimers. All methodologies have been adjusted for the maize

ADH system by Fischer (29). Mutants which tend to fall apart, bind Zn²⁺ poorly, or are unstable in the monomeric state are readily identified.

Two-Dimensional Immunoelectrophoresis and Other Anti-ADH Procedures. If an *Adh1* mutant specifies an inactive, nondimerizing polypeptide which is antigenically active, methods have been developed for its detection. Schwartz (30) discovered that the ADH-antibody complex has residual ADH activity. Capitalizing on this fact, Schwartz developed a sensitive two-dimensional immunoelectrophoresis technique. Since ADH1·ADH1 is relatively easy to purify to near homogeneity (4) all techniques which assay antigen-antibody precipitate directly are also applicable.

Incorporation of Radioactive Precursors into ADH1·ADH1. This laboratory rarely uses immunological procedures since Sachs' recent studies (Martin M. Sachs, manuscript submitted) on the selective synthesis of ADH during anaerobic treatment of primary roots. If a single maize seedling is subjected to N₂ atmosphere while its primary root is immersed in [¹⁴C]-leucine, the label is incorporated into only five proteins banded on native, slab, polyacrylamide gels. Figure 4 is an x-ray film autoradiograph of a slot from such a dried native gel; ADH1·ADH1 band is marked. Sachs' methods allow us to detect all mutant ADH1 product, including fragments, given that they are not quickly turned over by the cell. Further, rates of ADH synthesis supported by a mutant allele may be measured directly.

Peptide Maps and Sequencing. Felder, Scandalios, and Liu (4) have estimated that ADH1 polypeptides contain 32 lysines and arginines; the estimated maximum number of tryptic peptides is 33. Peptide maps are now in progress; no part of maize ADH1 has been sequenced, and terminal amino acid data have not been published.

In the case that mutant alleles of *Adh1* appear to specify nonmutant polypeptides, additional tests may be employed. Several of our underproducer and overproducer *Adh1* mutants induced by accelerated neon-20 are of this regulatory type.

Organ Specificity of Mutant Expression. A mutant may be over- or under-expressed in one organ but not in another. James C. Woodman, in this laboratory, has shown that several *Adh1* variants and a mutant are underproduced in one organ but reciprocally overproduced in another; this phenomenon has been called the "reciprocal effect" (31). I have compiled some relevant citations on the subject of organ-specific, specific gene variants and mutants in a recent publication (16).

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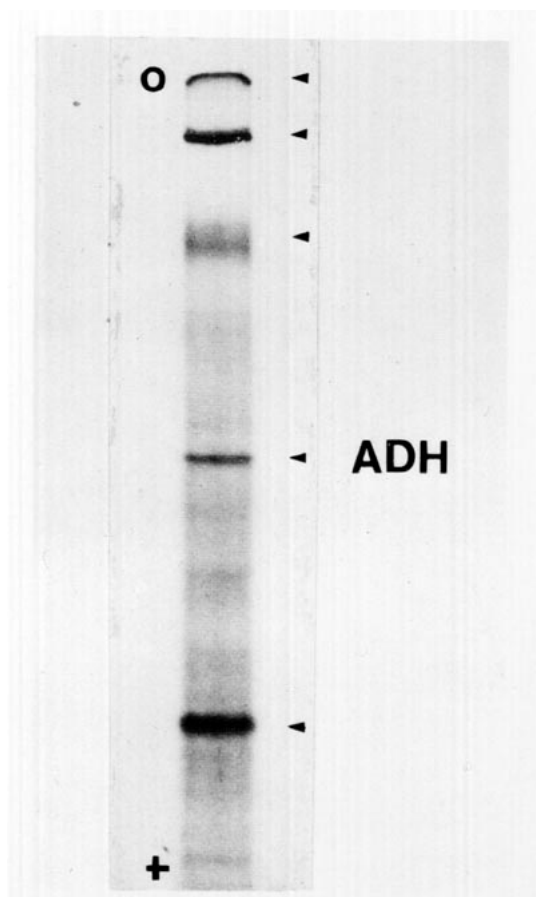


FIGURE 4. Autoradiograph of one slot from a dried, native, polyacrylamide gel slab showing [^{14}C]-leucine incorporation into five major anaerobic proteins (\blacktriangle), including ADH1-ADH1 (ADH). The sample was total, soluble protein from anaerobically-treated primary roots. Data of Sachs and Freeling (unpublished).

abounds with examples of unstable alleles, especially involving color loci (18, 19, 32). We now have Ne^{10+} -induced *Adh1*⁻ alleles which transmit "derivative" alleles. The derivatives express themselves to a flexible degree: some are normal-appearing while other derivative alleles are nearly inactive (15 unpublished data). Stability data are obtained by allozyme ratio determinations of back-crossed progeny where the maternal and paternal *Adh1* alleles are electrophoretically distinguishable (Fig. 3).

Dosage Effect or Compensation. Results on dosage effect or compensation by use of the *Adh1* gene system, and the models they have engendered, are among the most valuable contributions of *Adh1* (3, 33, 34), but are complex, hotly debated, and beyond the scope of this paper. *Adh1*-deficient mutant heterozygotes (+/-), when compared with normal siblings (+/+), may or may not have the same total ADH, depending on the nature of the mutant lesion.

Nucleic Acid Levels. Methodologies at the ADH-mRNA or ADH-DNA levels have not yet been established.

Maize *Adh1* as a Qualitative Monitor of Environmental Mutagens

No chemical mutagens or carcinogens have been used with our pollen technologies. Chemicals known to be environmental pollutants could be introduced into the developing corn plant in several ways: absorption through roots; injection into vascular system; injection into absorbent material implanted next to the immature tassel; or fumigation of the upper plant. The resultant mutants would be chemically selected, recovered and analyzed extensively in order to establish a spectrum of mutant types caused by a mutagen, and the ratio among these types; quantitative reversion or forward mutation assays might be run concomitantly.

I think that the maize *Adh1* system should be taken seriously as one of several ways to obtain data on the molecular nature of lesions caused by environmental mutagens, especially those suspected of causing birth defects and cancer. My argument is not complicated. Higher plants, like mammals, display the classical embryological phenomena of determination, competence and cell heredity, and their genome organization is typically "higher organism" (e.g. cotton) (35). No animal systems, with the exception of a few genes in *Drosophila* (e.g. *rosy* and *Adh*) even come close to the mutant analytical power afforded by the maize *Adh1* systems; however, extrapolations from the results of Illmansee and Mintz (36) on recovery of individual testicular carcinoma cells via allophenic mice looks promising. Perhaps maize *Adh1* will tell us eventually whether or not certain chemicals cause particular regulatory-type mutants while other chemicals confine their lesions to structural gene components. It is at least reasonable to entertain the notion that birth defects and cancer may be mediated preferentially by lesions in regulatory DNA. Chemicals specific to or acting preferentially on regulatory components would not be detected by point-mutant reversion monitor, and might be a great danger for society.

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